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Sequence Comparisons Link Toxic European Isolates of *Alexandrium tamarense* from the Orkney Islands to Toxic North American Stocks

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Summary

Twenty-one cultures of the *Alexandrium tamarense/catenella/fundyense* species- complex, isolated from a bloom at the Orkney Islands, north of Scotland, were examined morphologically and tested for toxicity using HPLC analyses. All cultures belong to the morpho-species of *A. tamarense* (Labour) Balech rather than *A. fundyense* Balech and are as toxic as the most toxic *A. fundyense* isolates from North America. A subset was genetically analysed using sequence data from the D1/D2 region of the LSU rRNA gene (656 nt). Genetic analyses indicated that these *A. tamarense* populations were related to North American isolates. At least three classes of the D1/D2 region were found in the cloned material. Sufficient base substitutions in the D1/D2 region preclude introduction into the Orkneys by human mechanisms (i.e., in ballast water or in shellfish stocks). The sequence analysis supports a dispersal hypothesis in recent evolutionary time of North American stocks of *A. tamarense* into Northern European waters via currents, possibly as part of the hypothesised original dispersal in the North Atlantic from the Pacific. In contrast, other Western European isolates of *A. tamarense*, whose D1/D2 region has been sequenced, are non-toxic and belong to a different gene pool.

Key words: *Alexandrium*; LSU rRNA; PSP; Toxicity.

Introduction

The number and intensity of toxic algal blooms, especially those involving paralytic shellfish poisoning (PSP), are increasing on a world-wide basis (7). Because of the severe economic and public health problems that these blooms impart, the causative organisms are currently the topic of national and international research programs. The genus *Alexandrium* contains several species responsible for PSP (7). The *Alexandrium tamarense/catenella/fundyense* species complex, in particular, has been extensively investigated. Genetic analyses of the SSU and LSU rRNA genes have shown that the evolutionary lineages within this complex reflect the geographic isolation of ancestral populations and not the morphotypes assignable to *A. tamarense, A. catenella* and *A. fundyense* (see references in 17). Hypotheses have been put forward to explain the radiation of these different geographical lineages from ancestral populations (17). By comparing sequence similarity between lineages, it has been possible to identify populations that likely arose from dispersal events in recent geological time as well as those more modern and likely of human introduction (i.e., in ballast water or in shellfish stocks). For example, populations of *A. tamarense* from North American stocks have been traced to introductions into Japan and subsequently as cysts in ballast water into Australia (17).

Toxicity has been correlated with certain geographically distinct populations as determined by sequence comparisons. Western European stocks of the *tamarense* complex, isolated from Spain and the Tamar Estuary, U.K., lack the characteristic B gene associated with toxic populations and are known to be non-toxic, although one cultured strain has been reported as mildly toxic (see references in 15). In contrast, toxic blooms

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of *A. tamarense* have been recorded at the Orkney Islands (Scotland) since 1960, although one record of a toxic event from the last century exists with the causative organism unidentified (see references in 2). Toxic *Alexandrium tamarense* are reported from the Faeroes [13), the west coast of Scotland [12), and in Denmark [8). PSP reports exceeding quarantine levels are documented along the western coast o Ireland and both coasts of Scotland [19). None of the toxic populations of *A. tamarense* from these locations throughout Northern Europe have been investigated using molecular techniques to determine their genetic affinities.

During a recent bloom of *A. tamarense* in May 1997 at the Orkney Islands, 21 isolates were brought into culture and examined morphologically. The D1/D2 region of the LSU rRNA gene was sequenced from several clones and compared to published sequences from other *Alexandrium* spp. Toxicity of the isolates was determined by HPLC methods on board ship and in the laboratory.

Material and Methods

Clonal isolations were made from samples taken at Station 57 of the 1997 cruise of the RV Heincke (58°N49°N), 02°30'W) and grown in F/2 media (Sigma). Specimens were prepared for SEM [14] and examined with a Zeiss DSM 940A scanning electron microscope. Isolates were tested for their toxicity using the extraction and HPLC methods detailed in [9] with toxin standards obtained from the NRC of Canada and the EU. These measurements were performed on board ship and again after return to the laboratory. Toxin profiles were compared with previously published toxin profiles from other toxic strains known from North America.

DNA extractions were performed on 18 isolates using a modified CTAB protocol [4] from samples preserved in 95% EtOH. The D1/D2 region of the LSU rRNA was amplified using PCR (polymerase chain reaction) with D1R forward and D2C reverse primers [16] following the PCR conditions given in [3] using Amplitaq polymerase (Perkin Elmer). Multiple amplifications of the D1/D2 region from individual isolates were pooled for automatic sequencing with the LiCor automatic sequencer (MWG) using the Sequi-Therm kit (BIOZYM) following the manufacturer's instructions. The amplification primers, modified with an infra-red label for the LiCor system, were used for sequencing. Individual PCR reactions were cloned using the LigATor Kit (R & D Systems) and then sequenced.

The sequences of the D1/D2 region from the cloned material were aligned [11] with published sequences from *Alexandrium* species [16, 20). Phylogenetic analyses were performed using maximum parsimony (PAUP, 18) and neighbor-joining (PHYLIP, 6) analyses (100 bootstrap replications, 5); only the PAUP tree is shown.

Results and Discussion

Morphological and toxicity data (Fig. 1 and 2) are presented for BAH ME 182, which is the clone analysed most extensively using molecular genetic techniques, although all other isolates are similar. Morphological features are typical for *A. tamarense* and toxicity levels are comparable to those of the most toxic isolates of *A. fundyense* reported from the eastern coast of North America. BAH ME 182 has similar toxin profiles to the toxic strain CCMP 1719 from Canada [9] (Fig. 2).

Eight isolates from the Orkneys were sequenced from PCR reactions pooled for each isolate. Sequence

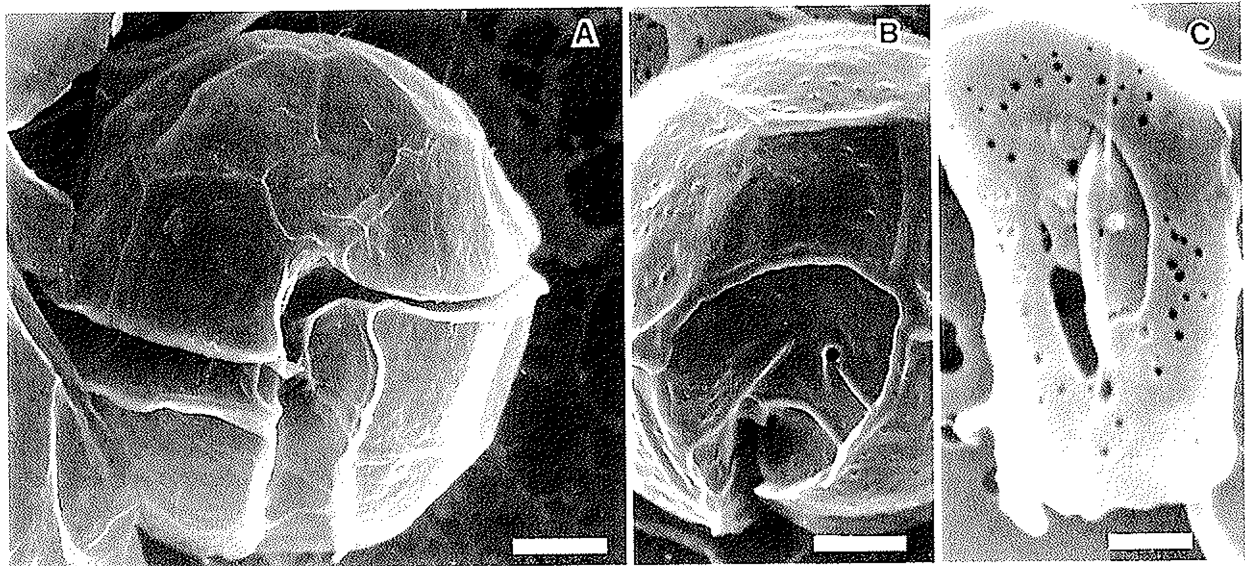

Fig. 1. SEM photomicrographs of *Alexandrium tamarense* St. 57 BAH ME 182. A Entire cell, scale bar = 5 µm. B. Detail of the sulcal region, scale bar = 5 µm. C. Detail of the apical pore plate, internal view, scale bar = 1 µm.

ambiguities were found that resulted in length heterogeneities indicative of multiple classes of the amplified D1/D2 region of the LSU rDNA (data not shown). The positions of sequence ambiguities were identical to those found in the North American isolates by Scholin et al. [16]. The amplified D1/D2 region from BAH ME 182 was cloned and sequenced to determine an unambiguous sequence for this region (656 nt). Three different sequence clusters were obtained, each containing different combinations of the two length classes of LSU rRNAs found by Scholin et al. [16] in their Newfoundland isolate from North America (Fig. 3). Clones BAH ME 182-1 & 7 were most similar to the longer of the length classes (AFNFA3.1) found by Scholin et al. [16]. Both contained the UUGUGGAAA motive at position 106 and 5 UG repeats at position 593, but clone 1 contained the inserted G at position 148 plus 12 base substitutions separating it from the Newfoundland isolate. Clone BAH ME 182-11 possessed the UUUGUGGAA motive at position 106, lacked the inserted G at position 148 similar to clone AFNFA3.2, but had 5 UG repeats at position 593. Two other sequences (clones 9 and 12) were intermediate between the two length classes found by Scholin et al. [16] and had the UUGUGGAAA, the inserted G at position 148, and 4 UG repeates at position 593. It is possible that some of these sequences may be chimeras, although the short length of the amplified fragment may preclude this. Other isolates are under investigation.

The cloned sequences were aligned with the *A. tamarense* species-complex data set [16] and analysed using maximum parsimony and neighbour-joining methods (Fig. 4). The isolates from the Orkneys are placed within the North American clade, specifically related to eastern seaboard populations, but are not identical to any North American isolate. This may pre-

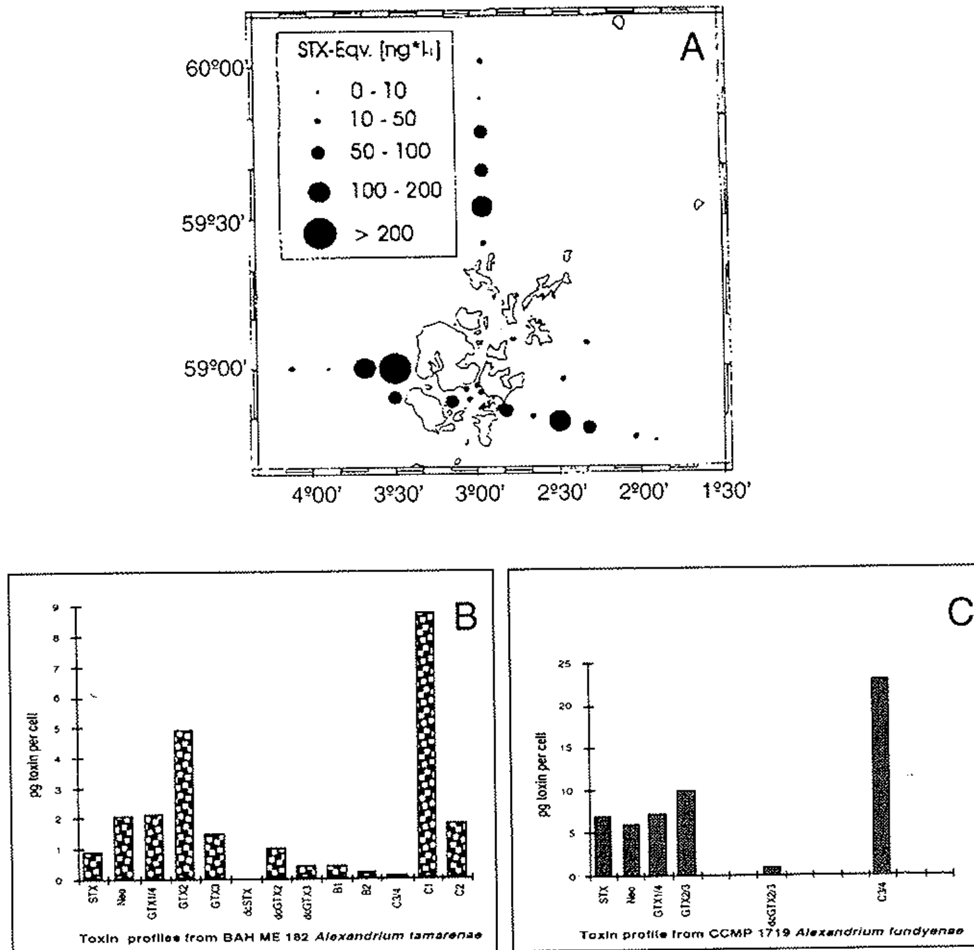

Fig. 2. (A) Map of the Orkney Islands showing the distribution of saxitoxin equivalents from samples taken around the island and measured on board ship. Histograms showing the toxin patterns as determined by HPLC analysis in (B) *Alexandrium tamarense* St. 57 BAH ME 182 and in (C) *A. fundyense* CCMP 1719 redrawn from [9]. Not all of the same toxins were measured in *A. fundyense*.

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POSITION
AFNFA3.1    UAAGUAAGUGGUGGAAAUUAAACCAAAUGGGAUAUCUUUAGUAAUUGCGAAUGAACAAGGAUAUGCUUAGCUUGACAAAUGGAGCUAUUGGCUUUGAAUU  AFNFA3.1
ORKNEY1     ....................................................................................................  ORKNEY1
ORKNEY7     ......................................................................................G.............  ORKNEY7
ORKNEY12    ....................................................................................................  ORKNEY12
ORKNEY9     .................................................................................S..................  ORKNEY9
ORKNEY11    ....................................................................................................  ORKNEY11
AFNFA3.2    ....................................................................................................  AFNF.3.2
PGT183      .................................U....................................................U..........G..  PGT183

POSITION        106                               148
AFNFA3.1    GUAUUGUGGAAAUGUAUUACCAACAGAGCUGCAGGUGCCAGCCUAUUGAAAUAAAGCGUCAAUGAGGGUGAGAAUCCUGUUUGUCAUGUGCAGCCCUUUG  AFNFA3.1
ORKNEY1     ..........................................A..........G.............................C................  ORKNEY1
ORKNEY7     ....................................................................................................  ORKNEY7
ORKNEY12    ....................................................................................................  ORKNEY12
ORKNEY9     ....................................................................................................  ORKNEY9
ORKNEY11    .....UGU.G..........................................................................................  ORKNEY11
AFNFA3.2    .....UGU.G..........................................................................................  AFNF.3.2
PGT183      ................CC...............A...Y.....AU......G.............................................C..  PGT183

POSITION
AFNFA3.1    UGCACGGUGUAUAUUUGCUGAGUCACACUCCUUGGCAUUGGAAUGCAAAGUGGGUGGUAAGUUUCAUGUMAAGGUAAACAUGCAAYUGAGACUGAUAGCA  AFNFA3.1
ORKNEY1     .....................................................................A...............C..............  ORKNEY1
ORKNEY7     .....................................................................AC..............U..............  ORKNEY7
ORKNEY12    .....................................................................A...............U..............  ORKNEY12
ORKNEY9     .....................................................................A...............U..............  ORKNEY9
ORKNEY11    .....................................................................AC..............U..............  ORKNEY11
AFNFA3.2    .....................................................................M...............Y..............  AFNF.3.2
PGT183      ...................................................................ACA........U......U..............  PGT183

POSITION
AFNFA3.1    CACAAGURCCAUGAGGGAAAUAUGAAAAGGACUUUGAAAAGAGAAUUAAAUGAGUUUGUAUUUGCUGAACACAAAGUAAACAGACUUGAUUUGCUUGGUG  AFNFA3.1
ORKNEY1     .......G......................UU..........U.........................................................  ORKNEY1
ORKNEY7     .......A..U.........................................................................................  ORKNEY7
ORKNEY12    .......A............................................................................................  ORKNEY12
ORKNEY9     .......A............................................................................................  ORKNEY9
ORKNEY11    .......A............................................................................................  ORKNEY11
AFNFA3.2    .......R............................................................................................  AFNF.3.2
PGT183      .......A....................................................................C.......................  PGT183

POSITION
AFNFA3.1    GGAGUGUUGCACUUGCUUGACAAGAGCUUUGGGCUGUGGGUGUAAUGAUUCUUUCUUUGCAUGCCAGUUUCUAUGUGUACAUCUGAUUACCUUUGCACAU  AFNFA3.1
ORKNEY1     ...................................................................C.........................A......  ORKNEY1
ORKNEY7     ....................................................................................................  ORKNEY7
ORKNEY12    ..........................................................................U.........................  ORKNEY12
ORKNEY9     ..........................................................................U.........................  ORKNEY9
ORKNEY11    ....................................................................................................  ORKNEY11
AFNF.3.2    ..........................................................................U.........................  AFNF.3.2
PGT183      A..U...A.UG............U..G...U...........A........................G..............U.................  PGT183

                                                                                                     593
POSITION
AFNFA3.1    GAAUGAUAAGUCUCCUGUGGGGGGUGGAUUGCAUGUGCAUGUAAUGAUUUGUGUUUUGAUAAAUGUGUCUGGUGUAUGUGUGUGUGUUCC-UGUGCUUGG  AFNFA3.1
ORKNEY1     ..........................................................................................-.......U.  ORKNEY1
ORKNEY7     ..........................................................................................-.........  ORKNEY7
ORKNEY12    ....................................................................................--....-.....C...  ORKNEY12
ORKNEY9     ....................................................................................--....-.........  ORKNEY9
ORKNEY11    ..........................................................................................-.........  ORKNEY11
AFNFA3.2    ....................................................................................--....-.........  AFNFA3.2
PGT183      .....GY..U.U.....C..........................................G................C...U.U......A....U...A  PGT183

POSITION
AFNFA3.1    GGAUGCUUCCUUCCUUGGACUUACAAGCCCUGACACACACAUGCUGGCAAAAUGCUUCUGCUUGACCCG  AFNFA3.1
ORKNEY1     .....................................................................  ORKNEY1
ORKNEY7     .....................................................................  ORKNEY7
ORKNEY12    .....................................................................  ORKNEY12
ORKNEY9     .....................................................................  ORKNEY9
ORKNEY11    .....................................................................  ORKNEY11
AFNFA3.2    .....................................................................  AFNFA3.2
PGT183      ..U...............G......................AU...........U..............  PGT183
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Fig. 3. Nucleotide sequences of the D1/D2 region from five clones amplified from *Alexandrium tamarense* BAH ME 182 (labelled Orkney 1, 7, 9, 11, 12) as compared with the two length classes of the Newfoundland isolate from North America (labelled AFNA3.1 and 3.2) and the PGT183 isolate from Western Europe [16]. Three diagnostic positions are numbered in the alignment.

clude them from being a recent introduction into the Orkneys from human activities, such as ballast water transport or translocated shellfish stocks. It is more likely that the populations were separated in recent evolutionary time.

Scholin et al. [17] have hypothesised that Atlantic populations of the *A. tamarense* species complex could represent populations that arose as Pacific populations of the species complex invaded the North Atlantic in the Pliocene with the opening of the Arctic Ocean through the Bering Strait. If the dispersal event into the Arctic Ocean was followed by a vicariant or dispersal event separating the ancestral populations into the eastern and western North Atlantic populations as climate and ocean circulation patterns changed, then molecular techniques should be able to uncover the direction of change. Although Scholin et al. [17] have hypothesised natural dispersal routes from the Pacific into the Atlantic between Northern Canada and Greenland (Fig. 5, route 1), it is equally likely that the populations could be distributed into the North Atlantic with the opening of the Atlantic between Greenland and Svalbard (Fig. 5, route 2). If the dispersal is through routes between Northern Canada and Greenland, then it is

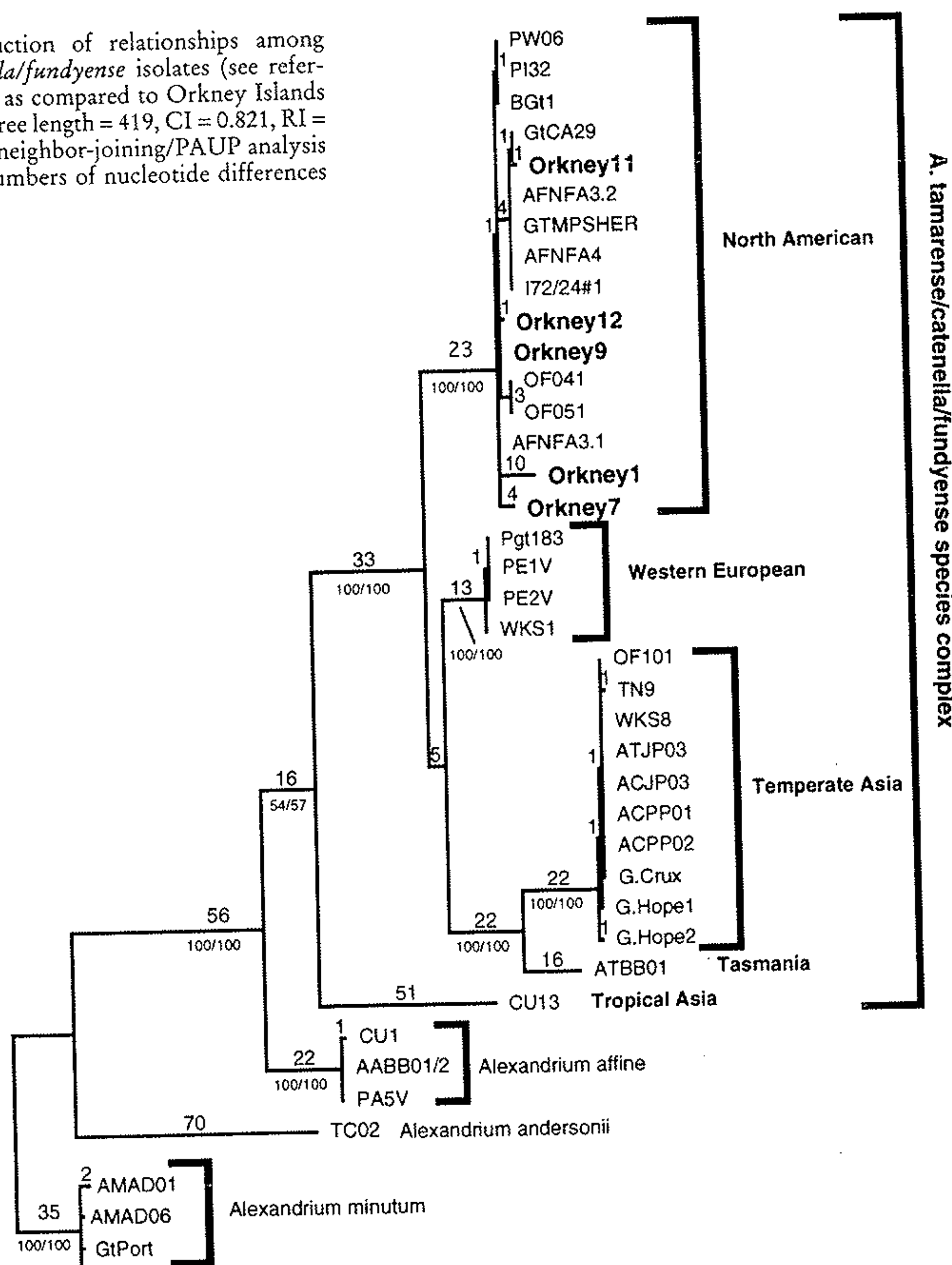

Fig. 4. Phylogenetic reconstruction of relationships among *Alexandrium tamarense/catenella/fundyense* isolates (see references in 14 for isolation details) as compared to Orkney Islands isolates using a PAUP analysis (tree length = 419, CI = 0.821, RI = 0.947). Bootstrap values from a neighbor-joining/PAUP analysis are presented below the line, numbers of nucleotide differences are above the line.

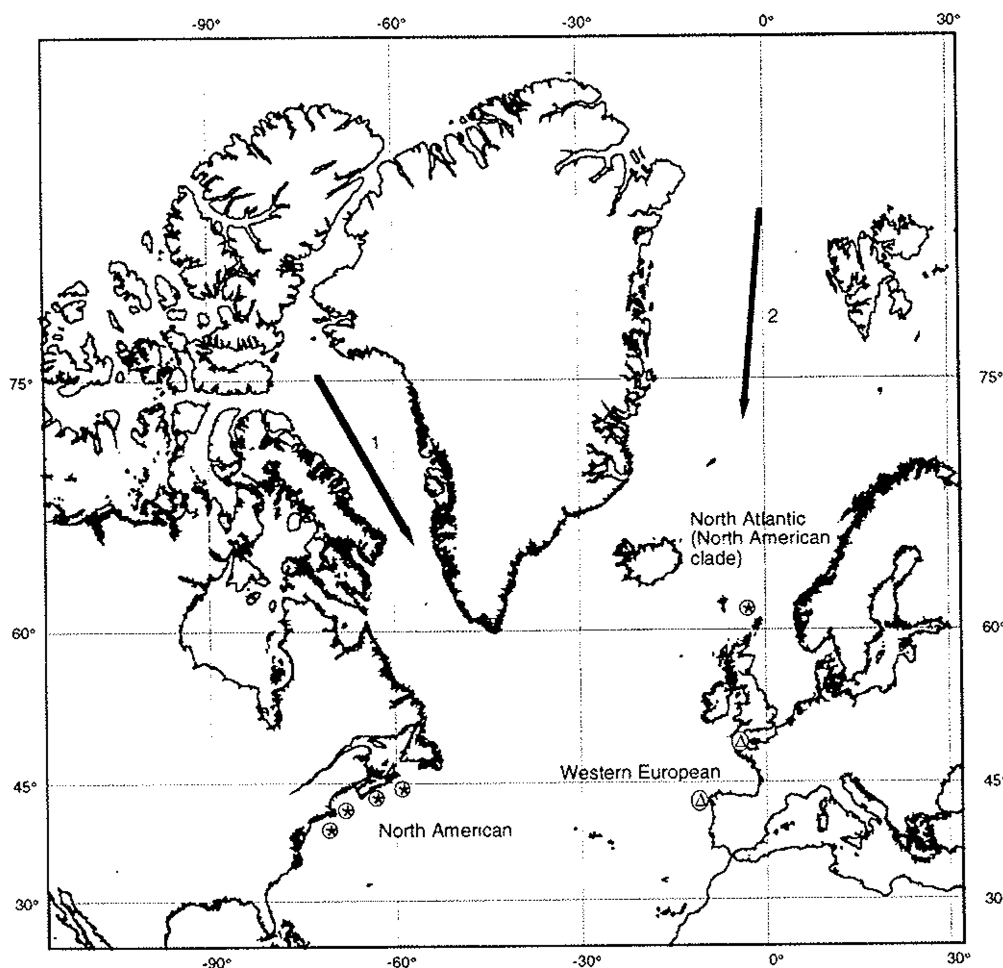

Fig. 5. Map of the North Atlantic showing [1] the hypothesized dispersal route of Scholin et al. [17] and [2] that hypothesized in this study. Encircled stars are the sampling locations for strains bearing the North American ribotype and the encircled triangles are the sampling locations for stains bearing the Western European ribotype.

possible that eastern North Atlantic populations may have been secondarily dispersed via currents, such as the Gulf Stream, from North America to Northern Europe (see references in 1, Fig. 5). If so, then the toxic *A. tamarense* populations from Northern Europe would be later divergences from the eastern seaboard populations of North America. If the dispersal route is through the passage between Greenland and Svalbard, then the toxic populations reported from Scandinavia and the northern British Isles could be ancestral to those along the eastern seaboard of North America. Unfortunately, there are very few base substitutions among D1/D2 regions from all of the isolates belonging to the North American clade making interpretation of the direction of change difficult. Some of the sequences may also be pseudogenes. A faster evolving region of the genome should be studied to clarify genetic relationships among the taxa and to reconstruct the evolutionary history of the group to determine the route of dispersal of the toxic populations into the North Atlantic from the Pacific.

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In Memoriam: André Hollande (1913–1998)

Professor Hollande in conversation with J. de Jonckheere (l.) and J. B. Jadin (r.).

On the 29 January 1998, André Hollande died in Verriéres-de-Buisson near Paris. He was known by many protistologists as the senior editor of *Protistologica*, sometimes feared, but always anxious to promote the quality of the aforesaid periodical initiated by Pierre de Puytorac. For those who were fortunate enough to known him, he was above all a naturalist of endless knowledge endowed with a profound curiosity with regards to the mysteries of life.

He was born on the 26 december 1913 in Nancy where his father was a professor at the Faculty of Pharmacy. André Hollande was brought up from childhood in scientific surrounding. He was lucky enough to pursue, during his studies of medicine and Science at the University of Montpellier, the teachings of famous masters such as E. Chatton and P. P. Grassé. From that stems all the preparation for the long and fruitful career of Protistologist for which he was famous in France and abroad. He was the student of P. P. Grassé whom he followed to Clermont-Ferrand, where he became his assistant in 1936, and later in 1938, in Paris at "La Sorbonne". His thesis on "Cytological and Biological studies of some free flagellated organisms, Volvocales, Cryptomonadines, Euglenids and Proteromonadines" was accomplished and presented in 1942.

Nominated "Maitre de Conférences" at the University of Algiers in 1945, he developed, together with Jean Cachon, a much appreciated school of Protistology. In 1952, he was promoted Professor and became the Director of the laboratory of Cell Biology. He stayed there until 1962 when he was obliged to return to France, due to the trouble which accompanied the independence of Algeria. He returned to Paris where he lectured at the University Pierre et Marie Curie and on the Bd Raspail in the "Laboratoire des Etres Organisés" founded by P. P. Grassé. He directed the department or Protistology which will become associated with the C.N.R.S. as E.R.A. 326. There he remained until his retirement. He was named emeritus professor in 1983. Three aspects marked the scientific career of André Hollande.

– As a man of the earth appreciating the fauna and flora, having learnt (very young, thanks to walks in the south of France) to distinguish various plants and ani-

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